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LEISHMANIA VECTOR SURVEILLANCE

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James C. McAvin

MAJ Joshua Bast, Ph.D.

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Entomology Department and Basic Science Laboratory, USAMRU-Kenya field station at Kisumu.

Clinical Research Division, CSPG/SGVUL, 59th MDW, 2200 Bergquist Drive,

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14. ABSTRACT

The Entomology Department and Basic Science Laboratory, USAMRU-K Field Station at Kisumu completed project activities through a resource sharing arrangement with the 59th MDW. Testing of the Leishmania epidemiology assay panel is completed for RAPID/JBAIDS-based detection assays, Leishmania genus (LEIS), visceral genotype (LVL), and human pathogenic (LHL). Test results provided valuable insight into the need to refine performance the LHL assay and the need to develop novel and unique sample preparation methodologies. Application for patent protection of this DoD intellectual property is underway.

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Clinical Research Division
59th Medical Wing, Lackland AFB, Texas

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Entomology Department and Basic Science Laboratory,
USAMRU-Kenya Field Station at Kisumu

Final Report

AFMSA O&M FY09 Project (FWH20090035): LEISHMANIA SURVEILLANCE AND DIAGNOSTIC CAPABILITY IN SUPPORT OF THE JOINT BIOLOGICAL AGENT IDENTIFICATION AND DIAGNOSTIC SYSTEM (JBAIDS)

&

AFMSA O&M FY10 Project (FWH20090195E): LEISHMANIA VECTOR SURVEILLANCE

February 07, 2013

Reporting Period: October 1, 2009 to February 7, 2013

Principal Investigator: Jim McAvin (Designated PI 22 July 2011)
Clinical Research Division, 59th Medical Wing, Lackland, AFB Texas

Report Prepared by:

Jim McAvin

&

Co-investigator: MAJ Joshua Bast, Ph.D.
Chief, Basic Science Laboratory,
United States Army Medical Research Unit -Kenya (USAMRU-K)
Field Station at Kisumu

Summary

During June 2012 to January 2013, the Entomology Department and Basic Science Laboratory, USAMRU-K Field Station at Kisumu completed project activities through a resource sharing arrangement with the 59th MDW. Testing of the *Leishmania* epidemiology assay panel is completed for RAPID/JBAIDS-based detection assays, *Leishmania* genus (LEIS), visceral genotype (LVL), and human pathogenic (LHL). Test results provided valuable insight into the need to refine performance the LHL assay and the need to develop novel and unique sample preparation methodologies. Application for patent protection of this DoD intellectual property is underway; McAvin, JC and RE Coleman: Methods, Manufactures, and Compositions Related to Leishmania. (Assignees: Secretary of the Army, Secretary of the Air Force, revised Jan 2013, reference AF Invention No. AFD 950, under review).

Continued transition of this technology for biosurveillance uses and pre-clinical test phase will be conducted under separate protocols. Proposed follow-on activities are directed at near-term commercialization of biosurveillance kits and ultimately transition and FDA clearance using the Next Generation Diagnostic System (NGDS).

Project Objectives (FY09):

1. Test and evaluate the current research-grade, freeze-dried (FD Leishmaniasis epidemiology panel assays on the RAPID and JBAIDS.

COMPLETED

2. Transition the freeze-dried Leishmaniasis epidemiology assay panel using the RAPID and JBAIDS.

COMPLETED

Transition for biosurveillance uses and pre-clinical test phase will be conducted under a separate protocol.

3. Validate performance of the *Leishmania* epidemiology assay panel and seek approval from the AFPMB and JBAIDS program manager for use in environmental surveillance.

COMPLETED

Testing is completed. The *Leishmania* genus (LEIS) and visceral genotype (LVL) RAPID-based assays are AFPMB approved for use in vector surveillance. Testing showed that the Old World human pathogenic *Leishmania* (LHL) assay requires re-optimization or potentially redesign. This work will be conducted under a separate protocol. A reformulated LHL assay and successful validation testing will potentially complete results needed for AFPMB and JBAIDS program manager approval of the RAPID/JBAIDS-based *Leishmania* epidemiology assay panel for vector (sand fly) and reservoir host (canids, rodents, and domestic animals) surveillance using the RAPID/JBAIDS.

4. Assess and evaluate *Leishmania* epidemiology assay panel performance on the RAPID and JBAIDS using clinical specimens and seek approval from the JPO/JBAIDS program manager as a qualified candidate assay for future FDA-clearance on the JBAIDS under a separate research proposal in the future. This very preliminary data additional funding will be required to complete the additional research necessary for FDA-clearance for human diagnostics and to sustain environmental surveillance operations. That JBAIDS FDA-clearance effort is not a part of this O & M proposal.

COMPLETED

Testing is completed. Visceral leishmaniasis (VL) patient sample test results support transition of the *Leishmania* assay panel for pre-clinical test phase. However, LHL assay performance and sample preparation procedures must be improved for clinical diagnostic use. Existing VL causative agent PCR detection tests show promise however improvement is needed in sensitivity and specificity for use in clinical diagnostics (1, 2, 3, 5, 6, 10, 11, 13, 15, 16). Currently, the most widely used detection tests are immunochromatographic (ICT) based technologies though there is increasing evidence of less than optimal performance in sensitivity and specificity (4, 14). Molecular and immunological tests require blood drawing and visceral aspiration typically from spleen and bone marrow. These procedures are highly invasive and do not address potential detection limitations presented from samples with *Leishmania* harbored at very low concentration.

The proposed 'Leishmania Detection Kit' includes novel and unique sample preparation methodology to assure efficacious diagnostic sensitivity (>90%). Slightly modified versions of the method allow for VL agent direct detection from urine, oral fluid, or conjunctival fluid. Fundamentally, the method involves serial microfiltration to concentrate *Leishmania* parasites followed by nucleic acid purification using conventional preformatted commercial kits. Sample processing requires less than one hour. The kit allows use with diverse real-time and conventional PCR analytic systems. Besides use for VL diagnosis the kit is readily adaptable for surveillance of reservoir hosts and vector. The kit has an important application in veterinary medicine. The invention provides for sensitive, specific, and rapid VL agent direct detection from non-invasively collected body fluids.

Kits will be developed for specific biosurveillance activities as well as clinical applications and alternative versions preformatted and categorized as 'high-tech' and 'low-tech' and "field-expedient".

The high-tech version of the kit requires use of real-time PCR instrumentation and relatively sophisticated molecular biology laboratory equipment and infrastructure. The primary applications are reference and clinical laboratory uses.

The low-tech version of the kit will be formatted for conventional PCR utilizing low cost reagents and consumables such as a precast, mini gel electrophoresis system. This technology is widely used by public health organizations and clinical laboratories in developing nations.

The field-expedient version of the kit requires use of deployable real-time PCR instrumentation and thermal-stabilized reagents. The intent of the field-expedient kit is support of far-forward military operations and humanitarian aid in situations of natural disaster.

This work will be completed under a separate protocol. Pending successful test results, the associated JPO/JBAIDS Molecular Assay Transition Package will be submitted to the JBAIDS Project Manager.

Project Objectives (FY10):

The FY10 project was follow-on to the FY09 project activities.

1. Provide scholarly and challenging Graduate Medical Education (GME) opportunities for residents, fellows and staff by imbedding them into the laboratory and field test and evaluation of current *Leishmania* epidemiology PCR assays for field expedient application as an assay panel on the RAPID and JBAIDS platforms.

Graduate Medical Education (GME) was not completed during the AFMSA O&M FY10 project funding period.

Note: under AFMSA RDT&E FY12 - FY13 funded projects a formal GME training program has been established by 59th MDW and AFRIMS investigators. Collaborators at USAMRU-Kenya remain open to providing GME opportunities. Travel restrictions are under assessment for regional locations within Kenya which will potentially allow GME TDY under a separate protocol(s).

2. Transition all the current *Leishmania* epidemiology PCR assays to one panel for field expedient use on the JBAIDS.

COMPLETED (see above)

3. Complete RAPID-based test and evaluation to support AFPMB approval of the RAPID-based *Leishmania* epidemiology assay panel for use in vector/vector-borne disease surveillance for field expedient use on the RAPID.

COMPLETED (see above)

4. Complete JBAIDS-based test and evaluation to support JBAIDS program manager approval of the JBAIDS-based *Leishmania* epidemiology assay panel for use in environmental (non-human) surveillance on the JBAIDS.

COMPLETED (see above)

5. Submit *Leishmania* epidemiology PCR assay panel to the JBAIDS program manager as a qualified candidate assays for future research targeted at getting FDA-clearance for human diagnostics on the JBAIDS under a separate research proposal.

COMPLETED

Testing under the FY09 and FY10 protocols is completed. Continued development and FDA clearance activities will be conducted under a separate protocol(s) pending funding. Pending successful test results, a JPO/JBAIDS Molecular Assay Transition Package will be submitted to the JBAIDS Project Manager. Under a separate protocol, the JBAIDS Molecular Assay Transition Package will be formatted as a pre-investigational device exemption (pre-IDE) document. The pre-IDE document will serve as the point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVDES) to obtain guidance and clarification on specific testing requirements for the eventual clearance of the '*Leishmania* Detection Kit'.

The pre-IDE document will describe the Kit and its intended use, proposed analytical testing and clinical evaluation strategies. The intent of pre-IDE guidance meetings are to ensure that proposed testing strategy is in line with current OIVDES thinking and is sufficient to support a pre-market notification application. The investigational device exemption (IDE) will allow the Kit to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification [510(k)] submission to FDA. Included in FDA OIVDES guidance meetings will be discussion on potential pre-IDE submissions for high throughput systems (HTS) and microarray systems. This will be accomplished under a separate protocol.

Funding for FY09 and FY10 projects was provided by the Air Force Medical Support Agency (AFMSA), Research & Acquisitions Directorate (SG5), Office of the Surgeon General (AF/SG), Falls Church, Virginia. This project was conducted by the Entomology Department and Basic Science Laboratory, USAMRU-Kenya Field Station at Kisumu and Clinical Research Division (CRD)/59th MDW. This project was executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19; AFI 25-201).

Products Completed

1. Technology transfer to a key DoD end-user of leishmaniasis biosurveillance technologies and clinical diagnostics.

2. Vector surveillance test results of the *Leishmaniasis* epidemiology panel assays LEIS, LVL, and LHL using the RAPID/JBAIDS.

3. Clinical sample test results of the *Leishmaniasis* epidemiology panel assays LEIS, LVL, and LHL using the RAPID/JBAIDS.

4. Technology transitioned to mid/late phase development toward DoD approved for biosurveillance use and pre-clinical testing.

Manuscripts

McAvin JC et al. *Leishmania* Detection from Clinical Samples Using Field-Deployable Real-Time PCR (in preparation).

McAvin JC, KI Swanson, AST Chan, M Quintana and RE Coleman. *Leishmania* detection in sand flies using a field-deployable real-time analytic system. Military Medicine 2012, 177(4):460-466.

Patents

McAvin, JC and RE Coleman: Methods, Manufactures, and Compositions Related to Leishmania. (Assignee: U.S.A., Secretary of the Air Force, revised Jan 2013, reference AF Invention No. AFD 950, under review).

Purpose

Leishmaniasis is considered as a military significant disease. The ability of medical personnel to accurately diagnose and recognize infectious disease threats in an operational environment is a high priority. The rapid identification of an infectious agent will allow for prompt, appropriate treatment, thereby minimizing morbidity and mortality. Additionally, knowledge about a specific infectious disease threat will allow for the implementation of prevention and control efforts to protect the fighting force.

Problem

Leishmaniasis is ranked among the top 40 diseases in the US Department of Defense global risk-severity index. Leishmaniasis is a zoonotic disease caused by obligate intracellular parasites of the genus *Leishmania* transmitted to humans through infected sand flies. Primary reservoirs are canids and rodents. The potentially fatal form of the disease, visceral leishmaniasis (VL), is caused by *Leishmania donovani* complex spp, *L. donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World. The non-fatal form of the disease, cutaneous leishmaniasis (CL), is caused primarily by *L. tropica* and *L. major* complex spp in the Old World and *L. mexicana* complex spp and subgenus *Viannia* complex *L. brazilliensis* and *L. guyanensis* spp in the New World.

There is no vaccine or prophylactic drug to use against leishmaniasis. One researcher has observed: "PCR based diagnosis for Visceral Leishmaniasis (VL), despite numerous published primers, remains far from being applied in the field (15). There is a need for early detection of *Leishmania* parasites, as an effective method of rapidly assessing risk and guiding treatment. Thus there is a need for rapid, sensitive and specific diagnostic assays. There is an additional need for *Leishmania* assay technology that is adaptable for surveillance and diagnoses in the field, under challenging environmental conditions.

Leishmaniasis force health protection can be enhanced by complete *Leishmania* epidemiology biosurveillance. Leishmanial diseases, the fatal visceral and non-fatal cutaneous (CL) forms, threaten deployed military forces. Prevention and control of *Leishmania* transmission are essential because in the absence of a vaccine or prophylactic drug the only means of protection is by preventing bites of infected sand flies and host abatement.

As a threat to combat effectiveness, DoD requirements have been established for the development and validation of detection technologies and deployment as an aid in point-of-care diagnostics and environmental surveillance operations. This study will address the critical need for field expedient assays to evaluate the complete epidemiology of leishmaniasis to support “real-time” preventative and control measures and provide a potential aid for human diagnostics.

Results

During 5 - 18 June, 2012 the 59th MDW Leishmania/RAPID system was transferred to collaborators at the Entomology Department and Basic Science Laboratory, USAMRU-K. The system consisted of a RAPID and freeze-dried TaqMan assays; *Leishmania* genus (LEIS), *Leishmania* visceral genotype (LVL), and *Leishmania* human pathogenic (LHL). In parallel with transfer activities the following were accomplished, leishmaniasis diagnostics and vector surveillance subject matter exchanges, experiment plan development, and follow-on proposal planning and coordination. Transfer activities were conducted at the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. The results of technology transfer, vector surveillance, and clinical sample testing follow;

Technology Transfer

Transfer activities included; system overview, review of previous RAPID-based *Leishmania* assay test results (LEIS, LHL, LVL, and LCL), LEIS, LHL, and LVL reagent QC testing, training in a field-expedient protocol for sand fly nucleic acid preparation, RAPID operations, freeze-dried reagent preparation, and experiment design. Results of QC testing are shown below (Figures 1-3).

On 18 June 2012, the system was transported to the Entomology Department and Basic Science Laboratory, USAMRU-K Field Station at Kisumu for the conduct and completion of the following;

1. LEIS, LHL, and LVL: clinical sample testing using a blind panel of $n \geq 10$ VL, $n \geq 10$ CL, and $n \geq 10$ clinically significant negative controls.

2. LEIS, LHL, and LVL: wild collected sand fly testing ($n \geq 10$ infected sand flies, $n \geq 10$ non-infected sand flies, and $n \geq 10$ relevant negative control organisms.

3. LHL: integrate validated assay in ongoing vector surveillance program. The LEIS and LVL assays are AFPMB approved for use in vector surveillance.

The 59th MDW investigator will;

4. LHL: pending successful test results seek AFPMB approval and JPO/JBAIDS qualification as a candidate for FDA clearance for use in human diagnostics.

5. LCL: primer and probe oligonucleotide re-design and computer-based analyses.

6. Pending successful test results author associated manuscripts.

7. Submit joint (59th MDW/USAMRU-K/AFRIMS/WRAIR) proposal to AF/SGR AFMSA for follow-on RDT&E, vector surveillance, and GME funding.

Figure 1. Results of Leishmania visceral genotype (LVL) freeze-dried assay QC testing

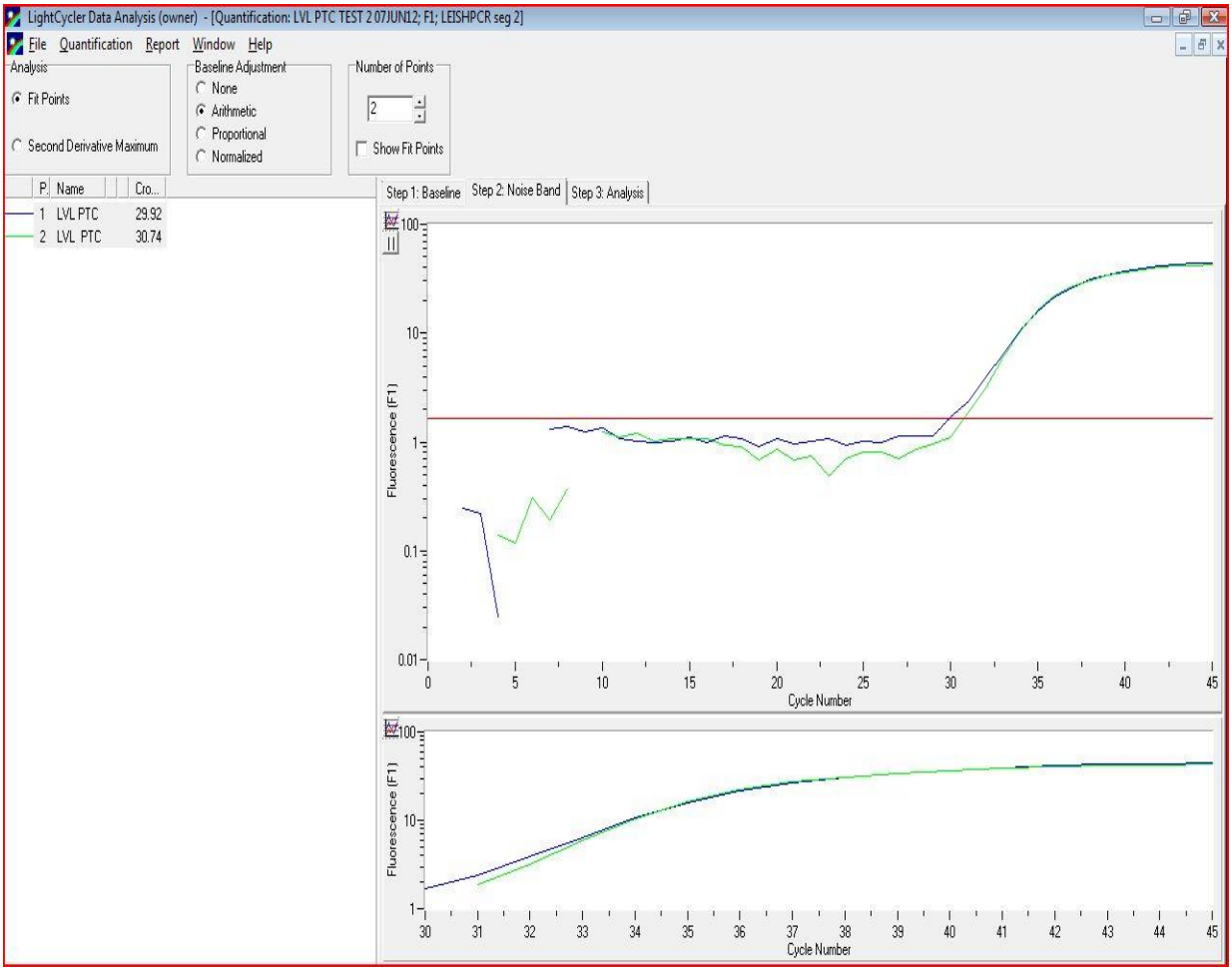


Figure 2. Results of Leishmania human pathogenic (LHL) freeze-dried assay QC testing

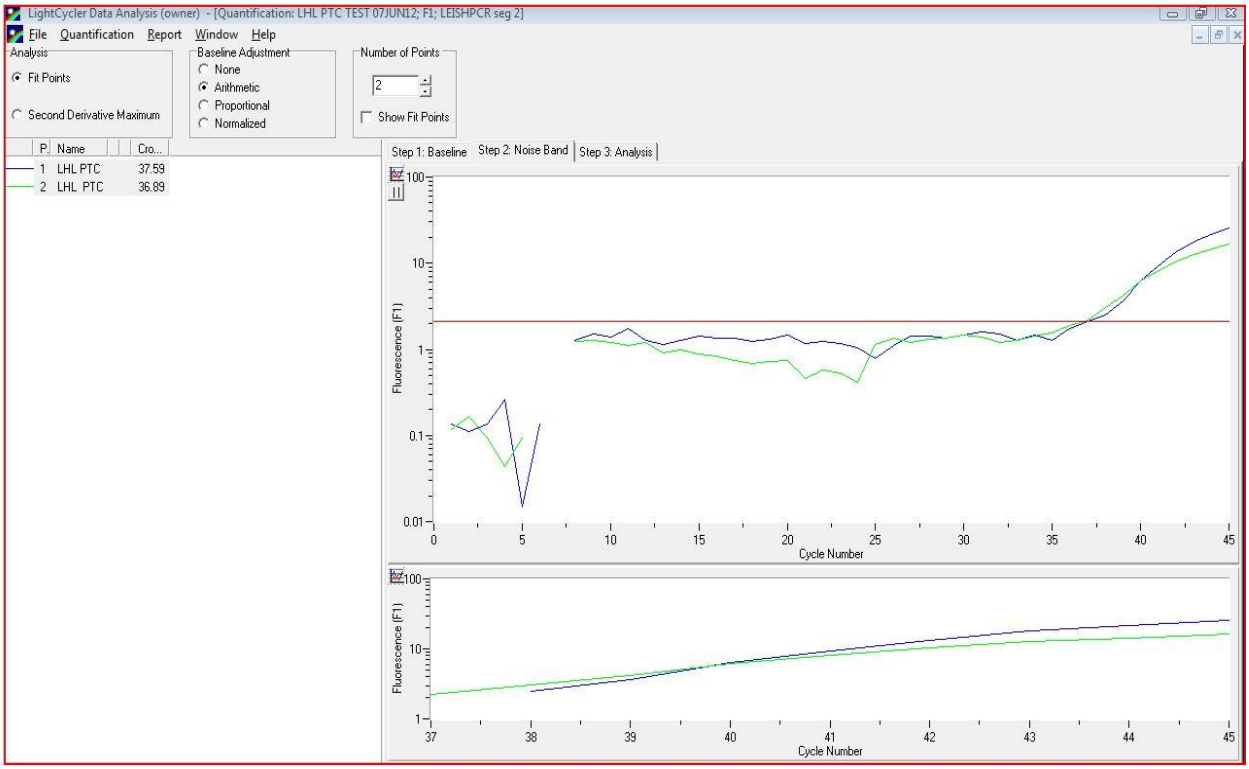
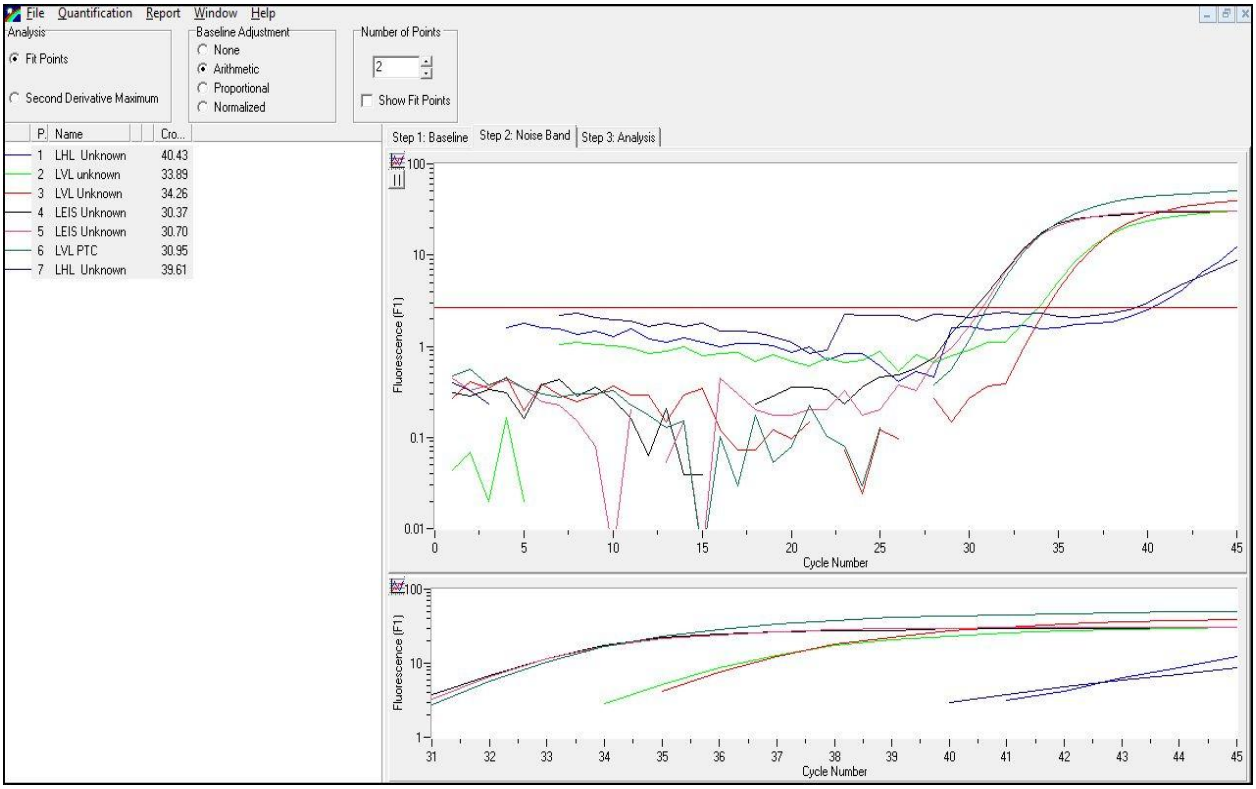


Figure 3. Results of Leishmania genus (LEIS) freeze-dried assay QC testing and LVL and LHL assay results using 1000 copies of *L. donovani* template



USAMRU-K Test Activities

Upon arrival at USAMRU-K RAPID system operations were confirmed and reagent QA/QC testing conducted (Table. 1). Performance of each assay was evaluated using the *Leishmania* RAPID system positive template control (PTC) reactions and control organism from culture. All of the assays worked well in testing using lab controls (culture DNA). Testing of the LHL assay using *L. tropica* from culture was repeated successfully. Each of the LEIS, LVL, and LHL assays were 100% sensitive and 100% specific in testing conducted with controls.

Table 1. Results of the Leishmania/RAPID QC testing.

Assay	**PTC**	***L. donovani***	***L. major***	***L. tropica***
		PCR Ct	PCR Ct	PCR Ct
LEIS (genus)	20.87	15.5	15.9	28.1
LVL (visceral genotype)	26.04	21.44	No amplification	No amplification
LHL (human pathogenic)	33.83	25.28	25.28	Did not Run

Vector Surveillance

Over a three year period USAMRU-K has conducted a sand fly surveillance study throughout the Horn of Africa (HOA). A total of 24 field trips were carried out at six sites in Kenya, four in Tanzania, and one in Ethiopia. Over 66,000 sand flies were sampled, over 11,000 identified and over 45,000 tested for *Leishmania* genus (GPI target) via conventional PCR. The sand flies were tested in pools consisting of ≤ 10 female sand flies each (n=4,471).

A total of 125 female sand fly pools have tested positive for Leishmania genus (~3%) by conventional PCR. Twelve of the pools (~10%) tested positive for pathogenic *Leishmania* by real-time PCR assays for VL causative agent *L. donovani* and CL causative agents *L. major* and *L. tropica*. The other 113 pools (~90%) positive for *Leishmania* but negative for pathogenic species are presumed to harbor non-pathogenic *L. tarentolae*. *L. tarentolae* is able to enter human macrophage cells and differentiate into amastigote-like forms however there is no clear evidence of efficient replication. The pathogenic *Leishmania* infection rate observed in sand flies correlates with the prevalence of leishmaniasis in the HOA region. The data from the study reported here supports previous test results and associated risk assessment conducted under the USAMRU-K HOA study.

Throughout the vector surveillance portion of this study each of the LEIS, LVL, and LHL assays were 100% sensitive and 100% specific in testing conducted with positive template control (PTC) and negative template control (NTC) reactions and control organism extracts (Tables 2). Compared to *Leishmania* genus (GPI target) real-time PCR the RAPID-based LEIS genus assay was 86% sensitive and 88% specific (Table 2). Compared to *L. donovani* (GPI target) real-time PCR the RAPID-based LVL assay was 80% sensitive and 87% specific. Compared to *L. donovani* (GPI target), *L. major* (GPI target), and *L. tropica* (GPI target) real-time PCR the RAPID-based LHL assay was 60% sensitive and 90% (6/44) specific.

Compared to previous LEIS and LVL analytic testing and sand fly surveillance results sensitivity and specificity rates observed in this study are much lower (McAvin et al. 2012). Concordant LEIS, LVL, and LHL assay results compared to the *Leishmania* genus (GPI target) suggest that various samples require further characterization. For example, samples 384, 388, and 418 were all negative by the *Leishmania* genus (GPI target) however LEIS, LVL, and LHL assays each reported robust fluorescence (Table 2). Moreover, samples 1405 designated as *L. donovani* was negative by each LEIS, LVL, and LHL assay in double-replicate testing and sample 313 designated as a negative by the *Leishmania* genus (GPI target) was positive by LEIS and LVL. Negative samples by the comparator test (1953, 495, and 1209) were reported as positive by LHL however each reported a Ct value of approximately 48. This is above the normal Ct 45 cut-off limit used and as such these results are considered negative. Based on previous testing and performance indicated in this study LHL sensitivity clearly needs improvement.

Note: calculations were based on; *Leishmania* negative samples = 45, *Leishmania donovani* = 4, *Leishmania major* = 1, and *Leishmania tropica* = 1.

Where the number of samples are;

a = True Positive; b = False Negative; c = False Positive; d = True Negative.

Sensitivity = (a / a + b)(100%)

Specificity = (d / c + d)(100%)

Table 2. Results of Vector Surveillance Using LEIS, LVL, and LHL Assays

3 July 2012

	Sample	Comparator Test (GPI)	LEIS Assay	LVL Assay	LHL Assay	Field-Site
1	492	*L. donovani* Ct 29.07	22.79	27.5	26.29	Isiolo
2	1405	*L. donovani* Ct 25.88				Garissa
3	396					West Pokot
4	198	*L. donovani* Ct 29.15	23.4	27.9		Wajir
5	MDH seline					Lab 1 sample
6	MDH 118					Lab 1 sample
7	L. major PBS 3		18.42			Ento L. major control
8	*L. donovani* dead		20.72	24.5	31.13	Ento L. donovani control
9	*L. tropica* PBS 4		capillary broke			Ento L. tropica ontrol
10	Genus PTC		25.11	_	_	LEIS PTC
11	LVL PTC		_	31.43	_	LVL PTC
12	LHL PTC		_	_	34.32	LHL PTC
13	NTC					

18 July 12

	Sample	Comparator Test (GPI)	LEIS Assay	LVL assay	LHL assay	Field-Site
1	82	Negative				Wajir
2	121	Negative				Wajir
3	184	Negative		42.48		Wajir
4	185	Negative				Wajir
5	190	Negative				Wajir
6	196	Negative		28.75		Wajir
7	*L. tropica* PBS 4		28.81			Ento L. tropica control
8	Genus PTC		31.76			
9	LVL PTC			37.51		
10	LHL PTC				36.65	
11	NTC					

24 July 12

	Sample	Comparator Test (GPI)	LEIS Assay	LVL assay	LHL assay	Field-Site
1	1908	Negative				Isiolo
2	1917	Negative			17.08	Isiolo
3	1923	Negative				Isiolo
4	1953	Negative			48.31 (Neg)	Isiolo
5	1962	Negative				Isiolo
6	313	Negative	26.85	37.91		Wajir
7	1405 (repeated)	Negative				Isiolo
8	384	Negative	18.7	23.38	24.62	Isiolo
9	388	Negative	21.51	25.8	24.62	Isiolo
10	PTC		27.62	28.73	31.37	JBAIDS PTC
11	NTC					

25 July 12

	Sample	Comparator Test (GPI)	LEIS Assay	LVL assay	LHL assay	Field-Site
1	411	Negative				Isiolo
2	418	Negative	42.8	19.99	20.5	Isiolo
3	490	Negative				Isiolo
4	495	Negative			48.31 (Neg)	Isiolo
5	500	Negative				Isiolo
6	506	Negative				Isiolo
7	517	Negative				Isiolo
8	388 (repeated)	Negative	21.51	25.8	24.62	Isiolo
9	PTC		28.23	28.89	29.03	JBAIDS PTC
10	NTC					

26 July 12

	Sample	Comparator Test (GPI)	LEIS Assay	LVL assay	LHL assay	Field-Site
1	523	Negative				Isiolo
2	548	Negative	35.19			Isiolo
3	1004	Negative				Isiolo
4	1209	Negative			48.31 (Neg)	Isiolo
5	1210	Negative			25.1	Isiolo
6	1380	Negative	38.97			Isiolo
7	1382	Negative				Isiolo
8	1383	Negative				Isiolo
9	1385	Negative				
10	Positive control		29.47	30.68	3.967	JBAIDS PTC
	Negative control					
1	1387	Negative				Isiolo
2	1389	Negative				Isiolo
3	1393	Negative				Isiolo
4	1394	Negative				Isiolo
5	1395	Negative		40.6		Isiolo
6	1396	Negative				Isiolo
7	1397	Negative				Isiolo
8	1399	Negative				Isiolo
9	1401	Negative				Isiolo
10	Positive control		30.75	31.18	9.879	JBAIDS PTC
11	Negative control					

Clinical Sample Testing

In the study reported here de-identified VL patient and healthy endemic control blood sample nucleic acid extracts were obtained from frozen storage. Blood samples were originally collected for a USAMRU-K clinical study conducted during 2007 and 2008 (1). Previously, subjects were classified as a confirmed VL case or a healthy endemic control based on clinical symptoms, ICT results, and microscopy of splenic aspirate. From each patient and control 200 µl of blood was collected and nucleic acid extract prepared using a commercial preformatted kit. Testing was conducted using PCR assays combined with ICT technology. By using two separate PCR/ICT-based systems simultaneously efficacious diagnostic sensitivity and specificity were achieved.

Testing conducted under the study reported here was approved by the institutional ethical committee of Kenya Medical Research Institute (KEMRI). Extracts were thawed and tested using LEIS, LVL, and LHL assays. Throughout the clinical sample testing portion of this study each all three assays were 100% sensitive and 100% specific in testing conducted with positive template control (PTC) and negative template control (NTC) reactions (Tables 3). Typical screen shots of RAPID analyses are shown below (Figures 4-6). Samples were blinded. The LEIS assay was used to determine the presence of VL causative agent infected samples versus healthy endemic controls. Compared to LEIS analyses, the LVL assay was 66% sensitive and the LHL assay was 54% sensitive. Performance in LVL and LHL diagnostic sensitivity was predictable because the sample processing methodology used in the 2007 - 2008 study was inappropriate for use with real-time PCR analysis.

The LEIS and LVL assays used in this study were finely optimized (McAvin et al 2012). The LEIS analytic LoD for *L. donovani* is 0.1 pg template (three genomic equivalents) per 20 µl reaction volume. The LVL assay LoD was established at 1.0 pg or 30 genomic equivalents. The LHL assay was established at 100 pg or 3000 genomic equivalents. Specialized sample preparation methodology is required to assure efficacious diagnostic sensitivity (> 90%). Total *Leishmania* from milliliter volumes of clinical sample must be captured and concentrated by ≥ 1e3 log concentration prior to nucleic acid extraction by commercial preformatted kits. For clinical samples with low *Leishmania* parasite concentration direct application of the standardized hundred microliter volume does not meet the detection limits of even finely optimized PCR assays. To achieve efficacious diagnostic sensitivity, clinical sample processing must be addressed.

The results of this study support this. Detection by the LEIS genus assay at a Ct value of ≤ 30 correlates well with LVL detection. Efficacious diagnostic sensitivity by LEIS and LVL analyses (and other well optimized PCR assays) can be achieved procedurally. Finer optimization of the LHL assay as well as our CL causative agent (LCL) detection assay with sample preparation methodology described in this report will potentially complete the *Leishmaniasis* epidemiology panel for biosurveillance and clinical uses.

Table 3. Results of VL Patient Sample Testing Using LEIS, LVL, and LHL Assays

Sample	Sample ID	LEIS Ct	LVL Ct	LHL Ct
1	ken 059	27.57	35.75	0
2	ken 081	0	Not Tested	Not Tested
3	ken 250	30.63	0	0
4	ken 206	27.79	32.92	0
5	ken 134	0	Not Tested	Not Tested
6	ken 151	36.71	0	0
7	ken 77	0	Not Tested	Not Tested
8	ken 249	29.79	33.85	0
9	ken 215	20.55	25.49	28.42
10	ken 061	26.68	33.62	0
11	ken 090	0	Not Tested	Not Tested
12	ken 084	0	Not Tested	Not Tested
13	ken 202	0	Not Tested	Not Tested
14	ken 132	0	Not Tested	Not Tested
15	ken 146	42.66	0	0
16	ken 248	25.69	31.82	0
17	ken 068	0	Not Tested	Not Tested
18	ken 226	22.97	25.9	34.74
19	ken 224	24.67	9.63	32.97
20	ken 087	0	Not Tested	Not Tested
21	ken 067	29.55	35.22	0
22	ken 249	27.78	33.85	0
23	ken 136	0	Not Tested	Not Tested
24	ken 135	0	Not Tested	Not Tested
25	ken 205	30.9	34.49	0
26	ken 073	35.58	0	0
27	ken 206	27.68	32.92	0
28	ken 217	17.88	18.39	31.48
29	ken 089	0	Not Tested	Not Tested
30	ken 088	0	Not Tested	Not Tested
31	ken 244	0	Not Tested	Not Tested
32	ken 134	0	Not Tested	Not Tested
33	ken 150	0	Not Tested	Not Tested
34	ken 074	0	Not Tested	Not Tested
35	ken 072	0	Not Tested	Not Tested
36	ken 208	32.81	0	0
37	ken 063	27.5	30.5	0
38	ken 082	35.99	0	0

39	ken 091	0	Not Tested	Not Tested
40	ken 246	30.69	0	0
41	ken143	36.01	0	0
42	ken 137	0	Not Tested	Not Tested
43	ken 073	43.15	0	0
44	ken 245	30.98	0	0
45	ken 064	30.96	0	0
46	ken 084	0	Not Tested	Not Tested
47	ken 083	0	Not Tested	Not Tested
48	ken 251	28.64	36.75	0
49	ken 145	0	Not Tested	Not Tested
50	ken 141	0	Not Tested	Not Tested
51	ken 144	38.47	0	0
52	ken 069	0	Not Tested	Not Tested
53	ken 251B	29.59	Not Tested	Not Tested
54	ken 067P	19.41	24.65	32.58
55	ken 060	31.48	0	35.82
56	ken 083	0	Not Tested	Not Tested
57	ken 148	0	Not Tested	Not Tested
58	ken 140	39.46	0	0
59	ken 142	0	Not Tested	Not Tested
60	ken 247	0	Not Tested	Not Tested
61	ken 080	0	Not Tested	Not Tested
62	ken 2640	22.71	28.87	0
63	ken 088	0	Not Tested	Not Tested
64	ken 147	0	Not Tested	Not Tested
65	ken 139	0	Not Tested	Not Tested
66	ken 070	0	Not Tested	Not Tested
67	ken 068	0	Not Tested	Not Tested
68	ken 262	17.9	22.7	30.11
69	ken 085	0	Not Tested	Not Tested
70	ken 086	0	Not Tested	Not Tested
71	ken 204	30.6	34.86	0
72	ken 133	0	Not Tested	Not Tested
73	ken 71	0	Not Tested	Not Tested
74	ken 77	0	Not Tested	Not Tested
75	ken T001	0	Not Tested	Not Tested
76	ken T002	0	Not Tested	Not Tested
77	ken T003	33.77	0	0
78	ken T004	0	Not Tested	Not Tested
79	ken T005	30.99	0	0
80	ken 160	0	Not Tested	Not Tested
81	ken 158	0	Not Tested	Not Tested

82	ken 152	0	Not Tested	Not Tested
83	ken 155	0	Not Tested	Not Tested
84	ken 157	0	Not Tested	Not Tested
85	ken 154	0	Not Tested	Not Tested
86	ken 159	0	Not Tested	Not Tested
87	ken 156	35.88	0	0
88	ken 157	0	Not Tested	Not Tested
89	ken 161	0	Not Tested	Not Tested
90	ken 162	0	Not Tested	Not Tested
91	ken 163	30.71	36.64	0
92	ken 165	0	Not Tested	Not Tested
93	ken 164	0	Not Tested	Not Tested
94	ken 166	0	Not Tested	Not Tested
95	ken 169	40.4	38.22	0
96	ken 168	0	Not Tested	Not Tested
97	ken 167	0	Not Tested	Not Tested
98	ken 170	0	Not Tested	Not Tested
99	ken 171	38.68	0	0
100	ken 172	0	Not Tested	Not Tested
101	ken 173	37.34	0	0
102	ken 175	29.88	0	0
103	ken B 006	0	Not Tested	Not Tested
104	ken B 005	0	Not Tested	Not Tested
105	ken 174	37.89	0	0

Figure 4.

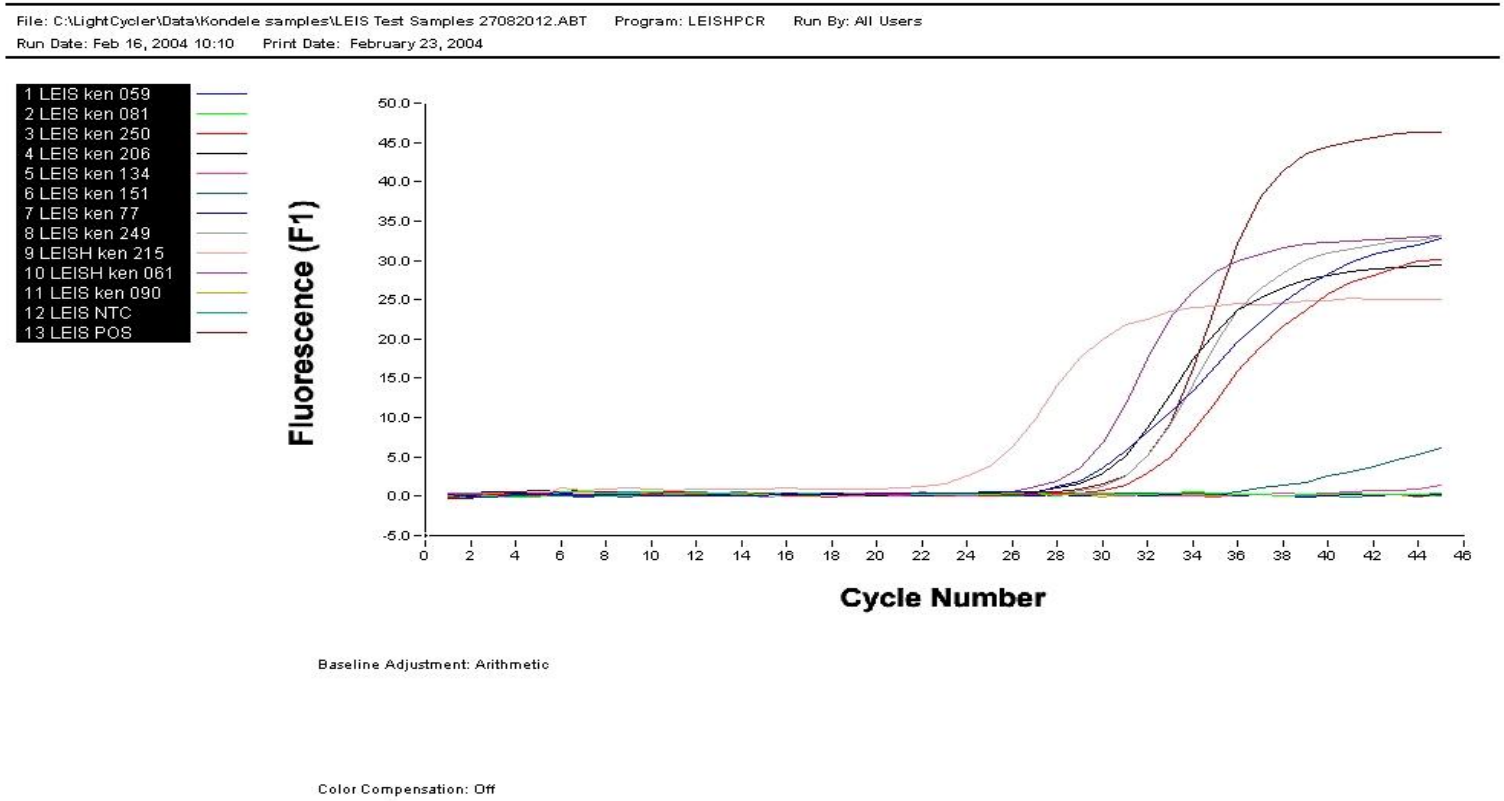

File: C:\LightCycler\Data\Kondele samples\LEIS Test Samples 27082012.ABT Program: LEISHPCR Run By: All Users
Run Date: Feb 16, 2004 10:10 Print Date: February 23, 2004
1 LEIS ken 059
2 LEIS ken 081
3 LEIS ken 250
4 LEIS ken 206
5 LEIS ken 134
6 LEIS ken 151
7 LEIS ken 77
8 LEIS ken 249
9 LEISH ken 215
10 LEISH ken 061
11 LEIS ken 090
12 LEIS NTC
13 LEIS POS
Fluorescence (F1)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Cycle Number
Baseline Adjustment: Arithmetic
Color Compensation: Off

Figure 5.

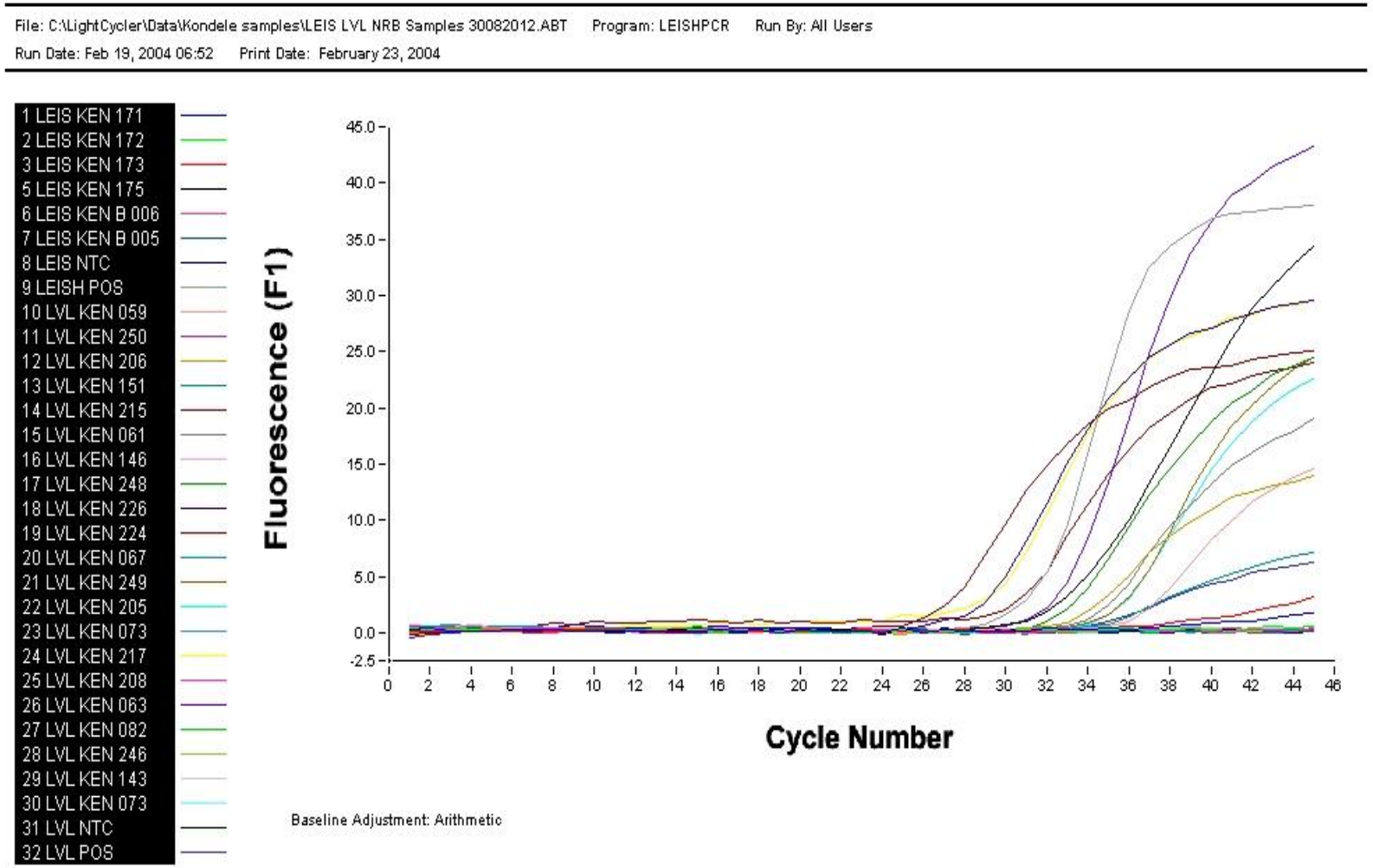
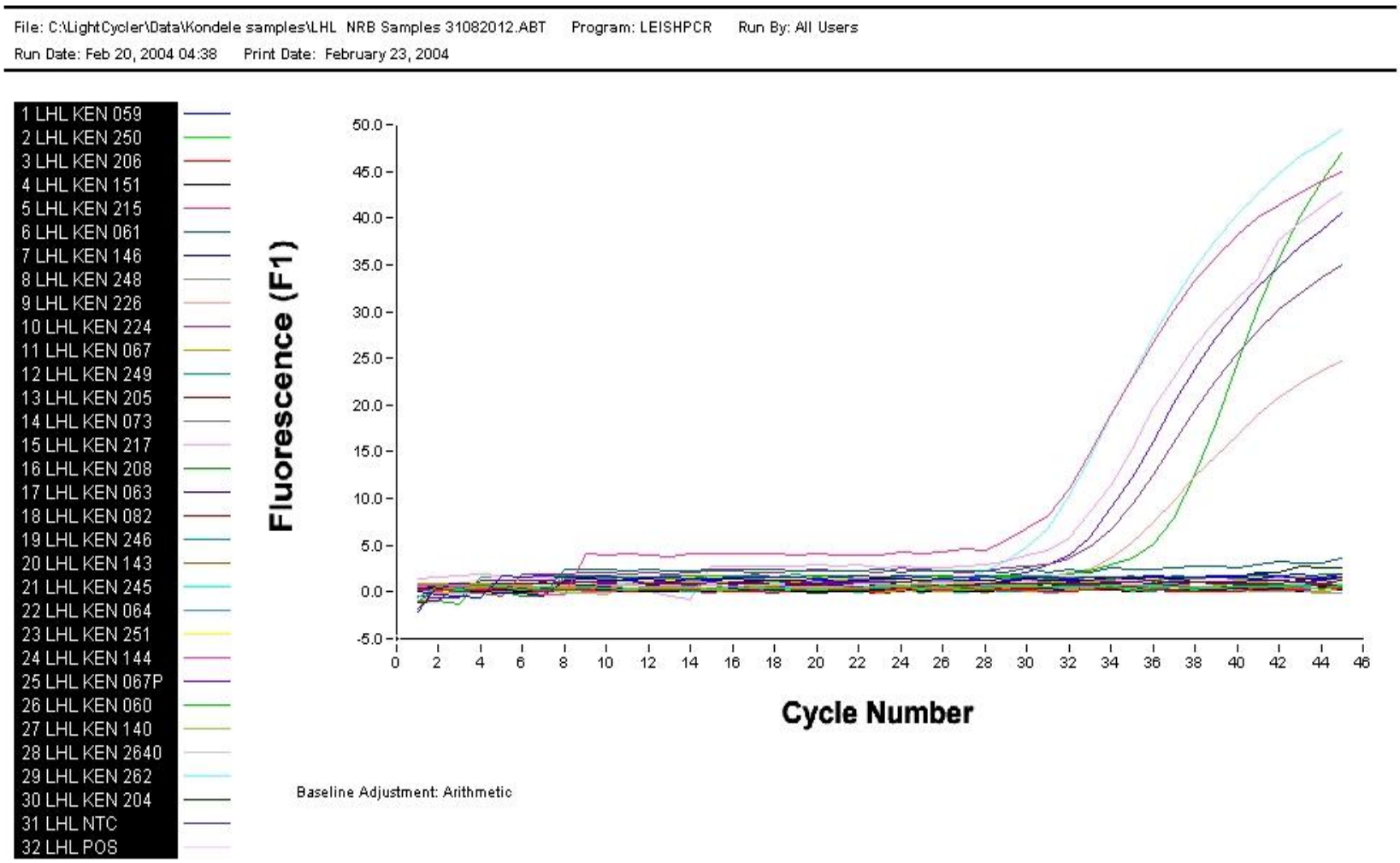

File: C:\LightCycler\Data\Kondele samples\LEIS LVL NRB Samples 30082012.ABT Program: LEISHPCR Run By: All Users
Run Date: Feb 19, 2004 06:52 Print Date: February 23, 2004
1 LEIS KEN 171
2 LEIS KEN 172
3 LEIS KEN 173
5 LEIS KEN 175
6 LEIS KEN B 006
7 LEIS KEN B 005
8 LEIS NTC
9 LEISH POS
10 LVL KEN 059
11 LVL KEN 250
12 LVL KEN 206
13 LVL KEN 151
14 LVL KEN 215
15 LVL KEN 061
16 LVL KEN 146
17 LVL KEN 248
18 LVL KEN 226
19 LVL KEN 224
20 LVL KEN 067
21 LVL KEN 249
22 LVL KEN 205
23 LVL KEN 073
24 LVL KEN 217
25 LVL KEN 208
26 LVL KEN 063
27 LVL KEN 082
28 LVL KEN 246
29 LVL KEN 143
30 LVL KEN 073
31 LVL NTC
32 LVL POS
Fluorescence (F1)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-2.5
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Cycle Number
Baseline Adjustment: Arithmetic

Figure 6.


File: C:\LightCycler\Data\Kondele samples\LHL NRB Samples 31082012.ABT Program: LEISHPCR Run By: All Users
Run Date: Feb 20, 2004 04:38 Print Date: February 23, 2004
1 LHL KEN 059
2 LHL KEN 250
3 LHL KEN 206
4 LHL KEN 151
5 LHL KEN 215
6 LHL KEN 061
7 LHL KEN 146
8 LHL KEN 248
9 LHL KEN 226
10 LHL KEN 224
11 LHL KEN 067
12 LHL KEN 249
13 LHL KEN 205
14 LHL KEN 073
15 LHL KEN 217
16 LHL KEN 208
17 LHL KEN 063
18 LHL KEN 082
19 LHL KEN 246
20 LHL KEN 143
21 LHL KEN 245
22 LHL KEN 064
23 LHL KEN 251
24 LHL KEN 144
25 LHL KEN 067P
26 LHL KEN 060
27 LHL KEN 140
28 LHL KEN 2640
29 LHL KEN 262
30 LHL KEN 204
31 LHL NTC
32 LHL POS
Fluorescence (F1)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Cycle Number
Baseline Adjustment: Arithmetic

Conclusion

During July 2012 to present extensive vector surveillance and clinical sample testing were accomplished. Results of VL patient sample testing and sand fly surveillance support previous data which clearly showed that the LVL assay is sensitive and specific. However, to assure that efficacious diagnostic sensitivity is achieved sample preparation methodology described in this report is required as well as improvement of LHL assay sensitivity and LCL redesign.

Results of VL patient sample testing and past analytic evaluations have shown the *Leishmania* Human Leishmaniasis species (LHL) primer and probe oligonucleotides to be specific for VL and CL agents however assay limit of detection (LoD) must be improved. In previous analytic testing, the LoD was established at approximately 1e3 *Leishmania* genome equivalents (100 pg template per 20 microliter reaction volume). The desired LoD is 1e1 genome equivalents (ge) or 0.10 pg template per 20 microliter reaction volume. The required LoD is 1e2 ge or 10 pg template per 20 microliter reaction volume. An increase in LoD of 1-2 logs (10X - 100X) of magnitude will be attempted by adjusting the salt and prime and probe concentrations of the current assay formulation. Reformulation to achieve an acceptable LOD has a high probability of success. If an acceptable LoD cannot be achieved by reformulation, primer and probe oligonucleotides redesign will be accomplished utilizing the original target sequences (A2 gene family), optimized using a standardized process, and LoD, sensitivity, and specificity testing conducted. Test results supported the above conclusion. The analytic LoD must be improved and the sample preparation methodology disclosed here will help assure efficacious diagnostic sensitivity. Past analytic test results showed *Leishmania* Cutaneous Leishmaniasis species (LCL) LCL1 and LCL2 primer and probe oligonucleotides require redesign. The A2 gene will remain the target of choice for CL agent specific primer and probe design.

A proof of concept study is required to demonstrate feasibility in principle. It remains to be verified that the *Leishmaniasis* epidemiology panel has the potential of being used. Successful validation testing and pre-clinical test phase will exponentially increase the value of this technology.

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The USAMRU-K laboratories conducted and completed vector surveillance and clinical sample testing well beyond the test plan agreed upon during technology transfer (June 2012). Thanks to the Clinical Research Division, 59th MDW for logistical support of technology transfer. Special thanks to the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand for hosting technology transfer activities.

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